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Biochemistry

# Identification and biochemical characterization of $p70^{TRK}$, product of the human *TRK* oncogene

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**ABSTRACT** ***TRK* is a human transforming gene generated in a colon carcinoma by a somatic rearrangement that fused a nonmuscle tropomyosin gene to sequences that shared extensive homology with members of the tyrosine-protein kinase supergene family. These sequences are likely to be derived from a transmembrane receptor gene whose putative ligand binding domain has been replaced by tropomyosin. In the present studies, we have expressed the entire coding sequences of the *TRK* oncogene as well as its protein kinase-related carboxyl-terminal domain in *Escherichia coli*. Antisera raised against these bacteria-synthesized TRK polypeptides has allowed us to identify the gene product of the *TRK* oncogene as a 70-kDa protein. Immunoprecipitates containing $p70^{TRK}$ have an associated protein kinase activity specific for tyrosine residues. Moreover, $p70^{TRK}$ is phosphorylated *in vivo* in serine (75%), threonine (20%), and tyrosine (5%) residues. Finally, immunofluorescence and cellular fractionation studies indicate that $p70^{TRK}$ is preferentially located in the cytoplasmic fraction.**

A large number of oncogenes are members of a complex family of genes encoding tyrosine-protein kinases (reviewed in ref. 1). Some of these genes code for cytoplasmic proteins that are often found associated with the inner side of the plasma membrane (e.g., viral genes v-*src*, v-*abl*). Others are transforming alleles of growth factor receptor genes. v-*erb*B, the oncogene of the avian erythroblastosis virus, is a truncated form of *EGFR*, the gene encoding the epidermal growth factor receptor (2). Similarly, v-*fms*, the oncogene of the McDonough strain of feline sarcoma virus (FeSV) is likely to be an allele of the gene encoding the receptor for CSF-1, a mononuclear phagocyte growth factor (3). Five additional oncogenes including v-*ros*/*MCF*3 (4, 5), v-*kit* (6), *NEU* (7), *MET* (8), and *TRK* (9) are also likely to be derived from transmembrane receptor loci.

The human *TRK* oncogene was generated by fusion of a nonmuscle tropomyosin gene with sequences derived from a new member of the tyrosine-protein kinase gene family likely to code for a transmembrane receptor (9). Southern blot analysis of DNAs isolated from the original colon carcinoma and from surrounding normal colonic tissue indicated that the genetic rearrangement that originated the *TRK* oncogene was specifically associated with tumor development (9). Three additional members of the tyrosine-protein kinase gene family have been implicated in the development of human neoplasia. Whereas the *ABL* oncogene appears to play an important role in the development of chronic myelogenous leukemia (10–12), the *EGFR* gene has been found to be amplified in some gliomas (13). Similarly, amplified *NEU* sequences have been identified in several human tumors, particularly in mammary adenocarcinomas (14–17), in which they appear to correlate with rapid relapse and may be used as a prognosis marker (17). In this report we describe the identification of the gene product of the *TRK* oncogene and its preliminary biochemical characterization.

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## MATERIALS AND METHODS

**Bacterial Expression.** Bacterial expression vectors pRC23 and pEV-vrf-1 containing the bacteriophage λ $P_L$ promoter were propagated in *Escherichia coli* DH5 cells previously transformed with the low-copy-number plasmid pRK248cIts that encodes a temperature-sensitive phage λ *c*I repressor (18, 19). The construction of the *TRK* expression plasmids pGM1 and pGM2 is outlined in Fig. 1. Bacterial cultures were grown at 30°C in NZY medium (22) containing 50 μg of ampicillin per ml to an optical density of 0.3 unit at 560 nm and were shifted to 42°C by rapid immersion in a 90°C water bath to allow expression from the $P_L$ promoter. After an additional incubation of 2 hr at 42°C, bacteria were collected, and their proteins were partially purified as described (20), analyzed by $NaDodSO_4$/PAGE, and stained with Coomassie brilliant blue.

**Cells and Antisera.** The 106-63 cell line is a third-cycle NIH 3T3 transformant derived from a human colon carcinoma DNA that carried the *TRK* oncogene (9). T227 cells are NIH 3T3 fibroblasts transformed with the Snyder–Theilen strain of feline sarcoma virus (ST-FeSV) (21). Antisera against bacteria-synthesized $p70^{TRK}$ and $p36^{TRK}$ polypeptides were prepared by subcutaneous inoculation of New Zealand White rabbits at 2-week intervals for 6 weeks with 50 μg of purified antigens. $p70^{TRK}$ and $p36^{TRK}$ polypeptides were isolated by preparative $NaDodSO_4$/PAGE.

**Cell Labeling, Immunoprecipitation, and Protein Kinase Assays.** Subconfluent cultures (10-cm dishes) were labeled with [$^{35}$S]methionine (50 μCi/ml; 1200 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) or with [$^{32}$P]orthophosphate (carrier-free) (1 mCi/ml, New England Nuclear) for 3 hr in methionine-free or phosphate-free Dulbecco's modified Eagle's medium, respectively. Cells were lysed and immunoprecipitated under conditions described previously (21). Protein kinase activity was carried out as described by Konopka and Witte (23). Phosphoamino acid analysis of electrophoretically purified or total [$^{32}$P]orthophosphate-labeled proteins was carried out as described (24).

## RESULTS

**Bacterial Expression of *TRK* Oncogene Products.** Identification of the gene product of the *TRK* oncogene required the generation of specific antibodies. We recently isolated a biologically active cDNA clone of the *TRK* oncogene, designated pDM10-1, that encompasses the entire coding sequences (9). We decided to utilize sequences derived from pDM10-1 to construct expression plasmids capable of directing the synthesis of the entire *TRK* oncogene product and of the tyrosine-protein kinase domain (Fig. 1). The first of these expression plasmids,

Abbreviations: FeSV, feline sarcoma virus; ST-FeSV, Snyder–Theilen strain of FeSV.

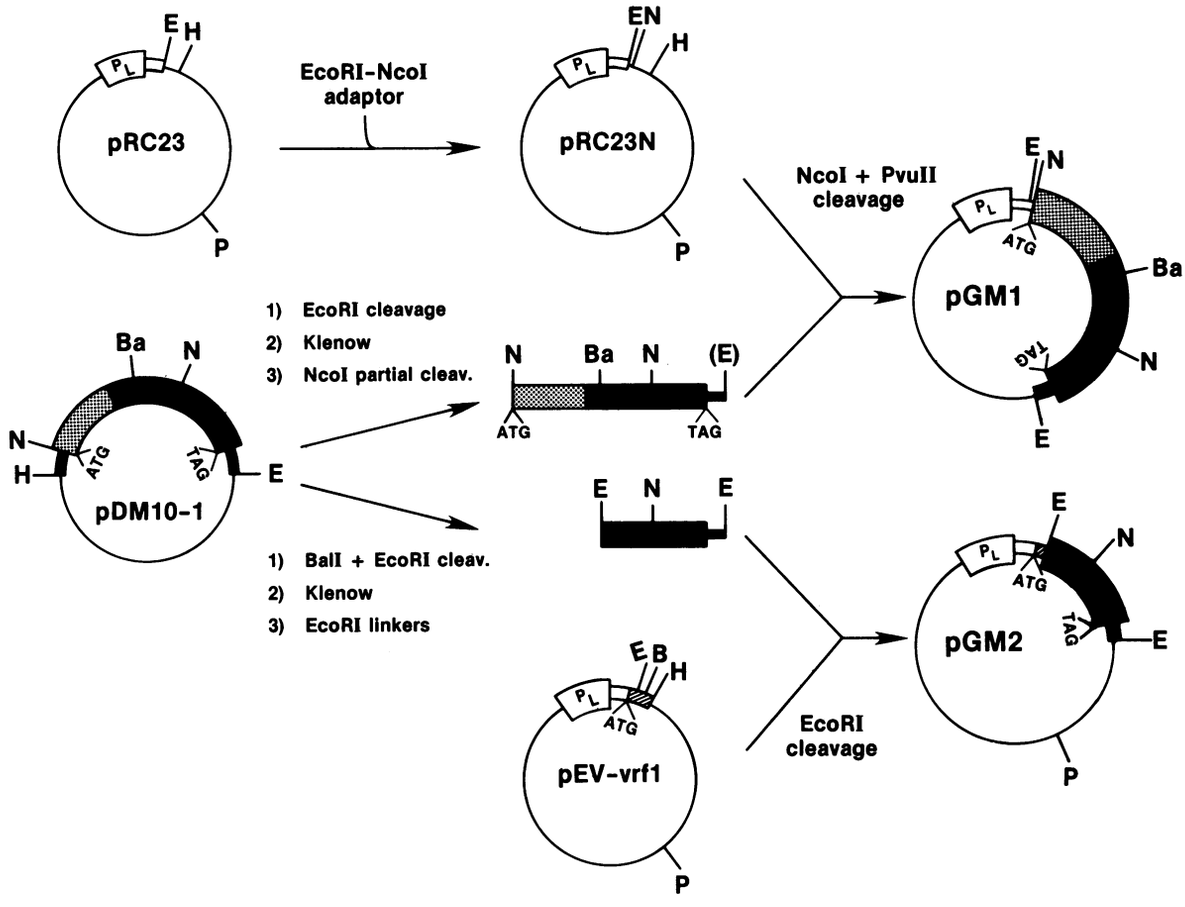


FIG. 1. Construction of the *TRK*-expression plasmids pGM1 and pGM2. The expression vector pRC23 (18, 19) was digested with *Eco*RI and ligated to a phosphorylated *Eco*RI–*Nco* I adaptor (5′ pAATTCCATGG-OH 3′) to generate an *Nco* I site next to the cloning *Eco*RI site. The resulting plasmid, pRC23N, was cleaved with *Nco* I and *Pvu* II and ligated to the 2.1-kilobase-pair (kbp) *Nco* I–*Eco*RI DNA fragment from pDM10-1, which encompassed the entire *TRK* coding sequences. Previously, the *Eco*RI site of this fragment was blunted in order to allow its ligation with the *Pvu* II site of pRC23N. To generate pGM2, a 1.25-kbp *Bal* I–*Eco*RI DNA fragment of pDM10-1 containing the tyrosine-protein kinase domain of the *TRK* oncogene was isolated, ligated with phosphorylated *Eco*RI linkers, and inserted at the unique *Eco*RI site of pEV-vrf-1 (18, 19) in the appropriate orientation. The stippled box represents the tropomyosin coding sequences of the *TRK* oncogene.

pGM1, was derived from pRC23 (18), a $P_L$ promoter-derived expression vector originally designed by Crowl *et al.* and previously utilized in our laboratory to produce proteins encoded by the Ha-*ras* oncogene (19). To generate pGM1, we first modified pRC23 by introducing an *Nco* I recognition site adjacent to the original *Eco*RI cloning site and the bacterial ribosomal binding site. The resulting plasmid, pRC23N, was digested with *Nco* I and *Pvu* II and ligated to a 2.1-kbp *Nco* I–*Eco*RI (blunted) DNA fragment of pDM10-1 containing the entire *TRK* coding sequences. This strategy allowed us to insert the ATG initiator codon of the *TRK* oncogene (encompassed within the *Nco* I cleavage site) a few nucleotides downstream from the bacterial ribosomal binding domain in order to ensure efficient translation (Fig. 1).

The second expression vector, pGM2, was designed to specifically express those *TRK* sequences that contain the tyrosine kinase-related sequences. For this purpose, a 1.25-kbp *Bal* I–*Eco*RI DNA fragment of pDM10-1 containing the 3′ half of the *TRK* oncogene cDNA was isolated. After adding *Eco*RI linkers, this fragment was inserted at the unique *Eco*RI site of pEV-vrf-1 (Fig. 1). pEV-vrf-1 is another $P_L$ promoter-derived expression vector that provides its own initiator codon followed by in-frame adaptor sequences containing *Eco*RI, *Bam*HI, and *Hin*dIII cleavage sites (18, 19).

pGM1 and pGM2 were used to transfect *E. coli* DH5 (pRK248cIts) that express the temperature-sensitive phage λ *c*I repressor capable of inhibiting transcription from the $P_L$ promoter at 30°C but not at 42°C. When shifted to 42°C, *E. coli* DH5 (pRK248cIts) cells transformed by either pGM1 or pGM2 expressed significant levels of polypeptides of 70 or 36 kDa, respectively (Fig. 2). The sizes of these polypeptides corresponded with the expected size of the *TRK* oncogene coding sequences present in pGM1 and pGM2 expression vectors. Both 70- and 36-kDa polypeptides were purified from the induced bacterial cultures by preparative $NaDodSO_4$/PAGE and injected into rabbits to elicit specific antibodies.

**$p70^{TRK}$ Is the Product of the *TRK* Oncogene.** Antisera obtained from rabbits immunized with the bacteria-synthesized p70 and p36 *TRK*-derived polypeptides were used to immunoprecipitate [$^{35}$S]methionine-labeled cell extracts of NIH 3T3 cells transformed by the *TRK* oncogene (106-63 clone). These antisera specifically recognized a protein of about 70 kDa in the *TRK*-transformed 106-63 cells (Fig. 3). This protein was not present in control NIH 3T3 cells (Fig. 3) or in NIH 3T3 cells transformed by a different oncogene such as human *HRAS1* (not shown). These results indicate that the p70 protein specifically identified in 106-63 cells is the product of the *TRK* oncogene. Interestingly, the anti-p70 antiserum did not contain significant levels of anti-tropomyosin antibodies (Fig. 3*A*), suggesting that most of the humoral response was elicited against the tyrosine-protein kinase-related domain of $p70^{TRK}$.

**$p70^{TRK}$ Has Tyrosine-Protein Kinase Activity.** The deduced amino acid sequence of the product of the *TRK* oncogene strongly suggests that this gene is a member of the tyrosine-protein kinase gene family (9). Therefore, we investigated

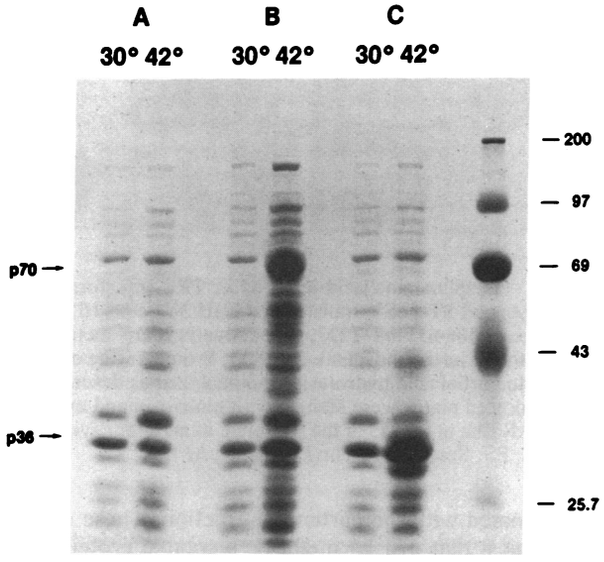


FIG. 2. Analysis of *TRK* oncogene products synthesized in *E. coli*. *E. coli* DH5 (pRK248cIts) cells transformed with pRC23N (lanes A), pGM1 (lanes B), or pGM2 (lanes C) expression vectors were grown and processed as described. Partially purified cell lysates (20) were subjected to $NaDodSO_4$/PAGE using 10.5% linear polyacrylamide gels and proteins visualized by Coomassie blue staining. Arrows indicate the migration of the $p70^{TRK}$ (lane B, 42°C) and $p36^{TRK}$ (lane C, 42°C) polypeptides encoded by the *TRK* oncogene sequences. Molecular mass markers, shown $\times 10^{-3}$, include myosin (200,000), phosphorylase b (97,000), bovine serum albumin (69,000), ovalbumin (43,000), and $\alpha$-chymotrypsinogen (25,700).

whether $p70^{TRK}$ molecules possessed this enzymatic activity. Immunoprecipitates containing $p70^{TRK}$ were capable of transferring [$^{32}$P]orthophosphate from [$\gamma$-$^{32}$P]ATP to $p70^{TRK}$ itself and to a lesser extent to the heavy chain of immunoglobulin IgG molecules (Fig. 4*A*). No such kinase activity was observed in parallel immunoprecipitates obtained by incubating control NIH 3T3 cells with the same anti-TRK antibodies. This protein kinase activity was dependent on the presence of divalent cations such as $Mn^{2+}$ or $Mg^{2+}$. $p70^{TRK}$, like the products of other mammalian tyrosine-protein kinase oncogenes, exhibited a preference for $Mn^{2+}$ ions (Fig. 4*A*).

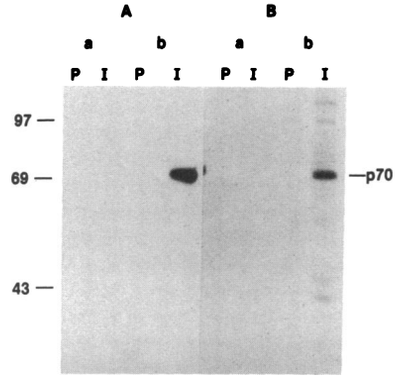


FIG. 3. Identification of the *TRK* oncogene product. [$^{35}$S]-Methionine-labeled cell extracts from normal NIH 3T3 cells (lanes a), and *TRK*-transformed NIH 3T3 cells (clone 106-63) (lanes b) were immunoprecipitated with preimmune (lanes P) or immune (lanes I) sera obtained from rabbits immunized with $p70^{TRK}$ (p70) (*A*) and $p36^{TRK}$ polypeptides (*B*) purified by preparative $NaDodSO_4$/PAGE from *E. coli* cells transformed with pGM1 and pGM2, respectively. Immunoprecipitates were analyzed as described (22). Gels were exposed to Kodak XAR films for 2 days. Molecular weight markers, shown $\times 10^{-3}$, were those described in the legend to Fig. 2.

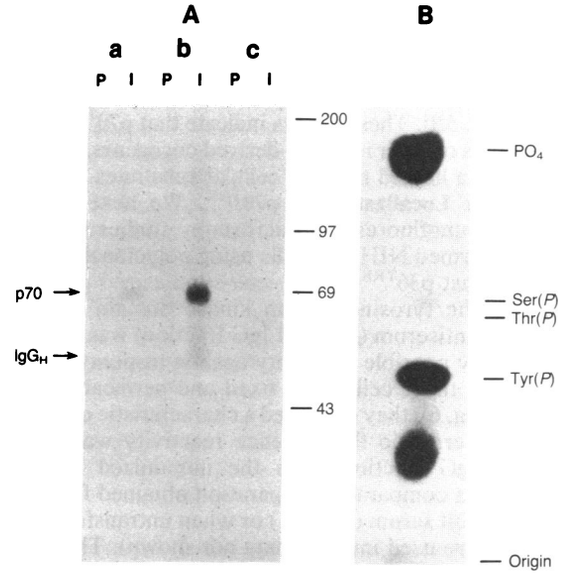


FIG. 4. Tyrosine-protein kinase activity in immunoprecipitates containing $p70^{TRK}$. (*A*) Protein kinase activity in immunoprecipitates of *TRK*-transformed NIH 3T3 cells. Extracts derived from *TRK*-transformed cells (clone 106-630) (lanes a and b) or normal NIH 3T3 cells (lanes c) were immunoprecipitated with preimmune (lanes P) or immune (lanes I) sera obtained from rabbits immunized with bacteria-synthesized $p70^{TRK}$ and analyzed for protein kinase activity as described (23). Gels were exposed at −70°C to Kodak XAR film with an intensifying screen for 24 hr. Arrows indicate the migration of the phosphorylated $p70^{TRK}$ (p70) and the heavy (H) chain of immune IgG molecules ($IgG_H$). Molecular weight markers, shown $\times 10^{-3}$, were those described in the legend to Fig. 2. (*B*) Phosphoamino acid analysis of *in vitro* phosphorylated $p70^{TRK}$. [$^{32}$P]-labeled phosphoamino acids were detected by autoradiography for 24 hr with an intensifying screen. Nonradioactive standards, including phosphoserine, phosphothreonine, and phosphotyrosine, were detected by ninhydrin staining.

The phosphate-acceptor amino acid residue of this *in vitro* kinase activity was next examined. Acid hydrolysates of [$^{32}$P]orthophosphate-labeled $p70^{TRK}$ were subjected to electrophoresis at pH 3.5 on cellulose-coated glass plates. Tyrosine was the main amino acid residue that became phosphorylated by the *in vitro* protein kinase activity present in immunoprecipitates containing $p70^{TRK}$ proteins (Fig. 4*B*). In addition to their autophosphorylating activity, immunoprecipitates containing $p70^{TRK}$ were capable of phosphorylating tyrosine residues of exogenously added substrates such as a mixture of calf thymus histones (data not shown). These results support the concept that $p70^{TRK}$ is indeed a tyrosine-protein kinase.

**Phosphotyrosine Residues in $p70^{TRK}$.** Phosphorylation of tyrosine residues is thought to play a major role in the regulation of the catalytic activity of tyrosine-protein kinases (1). Therefore, we investigated whether $p70^{TRK}$ was a phosphoprotein and, if so, whether it contained phosphotyrosine residues. For this purpose we labeled 106-63 cells with [$^{32}$P]orthophosphate and incubated them with antisera elicited against the $p70^{TRK}$ protein synthesized in *E. coli* cells. Phosphoamino acid analysis of steady-state [$^{32}$P]orthophosphate-labeled $p70^{TRK}$ molecules revealed serine (75%) and threonine (20%) as the major acceptor phosphoamino acid residues. However, a significant fraction (about 5%) of the radioactivity incorporated in $p70^{TRK}$ was identified as phosphotyrosine. These results open the possibility that tyrosine phosphorylation may play a role in the regulation of the tyrosine-protein kinase activity of $p70^{TRK}$.

We also determined whether NIH 3T3 cells transformed by the *TRK* oncogene possessed increased levels of phosphotyrosine residues. Neither NIH 3T3 cells (not shown) nor NIH 3T3

cells transformed by the *TRK* oncogene (106-63 cells, Fig. 5*A*) showed detectable levels of tyrosine phosphorylation. In contrast, ST-FeSV-transformed NIH 3T3 cells utilized as positive controls exhibited the expected increase in phosphotyrosine residues (Fig. 5*B*). These results indicate that $p70^{TRK}$, like the gene products of other receptor-derived oncogenes (24, 26, 27), may possess a limited range of cellular substrates.

**Subcellular Localization of $p70^{TRK}$.** We next conducted indirect immunofluorescent microscopy studies with 106-63 *TRK*-transformed NIH 3T3 cells using polyclonal antibodies elicited against $p36^{TRK}$, the *E. coli*-synthesized polypeptide containing the tyrosine-protein kinase domain of $p70^{TRK}$. This rabbit antiserum (purified IgG fraction) was selected to eliminate any possible reactivity against tropomyosin molecules. When these cells were fixed and permeabilized with methanol (Fig. 6), they exhibited a characteristic cytoplasmic staining pattern. No fluorescence reactivity was observed when the IgG fraction from the immunized rabbit was replaced by a comparable preparation obtained from preimmunized rabbit serum (Fig. 6*A*) or when untransformed NIH 3T3 cells were used instead (data not shown). These results indicate that $p70^{TRK}$ molecules are preferentially located in the cytoplasm of *TRK*-transformed NIH 3T3 cells.

To ascertain whether $p70^{TRK}$ was associated with membranous structures (e.g., endoplasmic reticulum) or was present in a soluble form, we submitted [$^{35}$S]methionine-labeled *TRK*-transformed 106-63 cells to fractionation into nuclear, nuclear membrane, cytoplasmic, and particulate fractions by established protocols (26). Fractions were incubated with anti-$p70^{TRK}$ antibodies, and the resulting immunoprecipitates were analyzed by $NaDodSO_4$/PAGE. Most (about 90%) of $p70^{TRK}$ was identified in the soluble cytosolic fraction (Fig. 7). Only a small amount (about 10%) of $p70^{TRK}$ cofractionated with the particulate fraction. These observations suggest that $p70^{TRK}$ might be a soluble protein. However, our results cannot rule out the possibility that under physiological conditions some $p70^{TRK}$ molecules become weakly associated with either membranes or other subcellular structures. Finally, iodination of intact 106-63 cells failed to label $p70^{TRK}$ molecules (data not shown), indicating that the small percentage of $p70^{TRK}$ associated with membranous structures is not anchored across the plasma membrane.

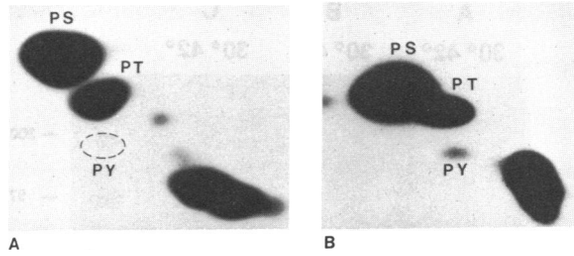


FIG. 5. Phosphoamino acid analysis of *TRK*-transformed NIH 3T3 cells (*A*) and ST-FeSV-transformed NIH 3T3 cells (*B*). Cells ($3 \times 10^6$; clones 106-63 and T227, respectively) were metabolically labeled with [$^{32}$P]orthophosphate for 18 hr. Proteins were extracted, subjected to partial acid hydrolysis, and analyzed as described (25). Electrophoresed plates were stained with ninhydrin and exposed to Kodak XAR film at −70°C in the presence of an intensifying screen for 2 days. PS, Ser(*P*); PT, Thr(*P*); PY, Tyr(*P*).

## DISCUSSION

*TRK* is a transforming gene generated in a human colon carcinoma by a somatic rearrangement involving nonmuscle tropomyosin sequences and a novel gene that exhibits fea-

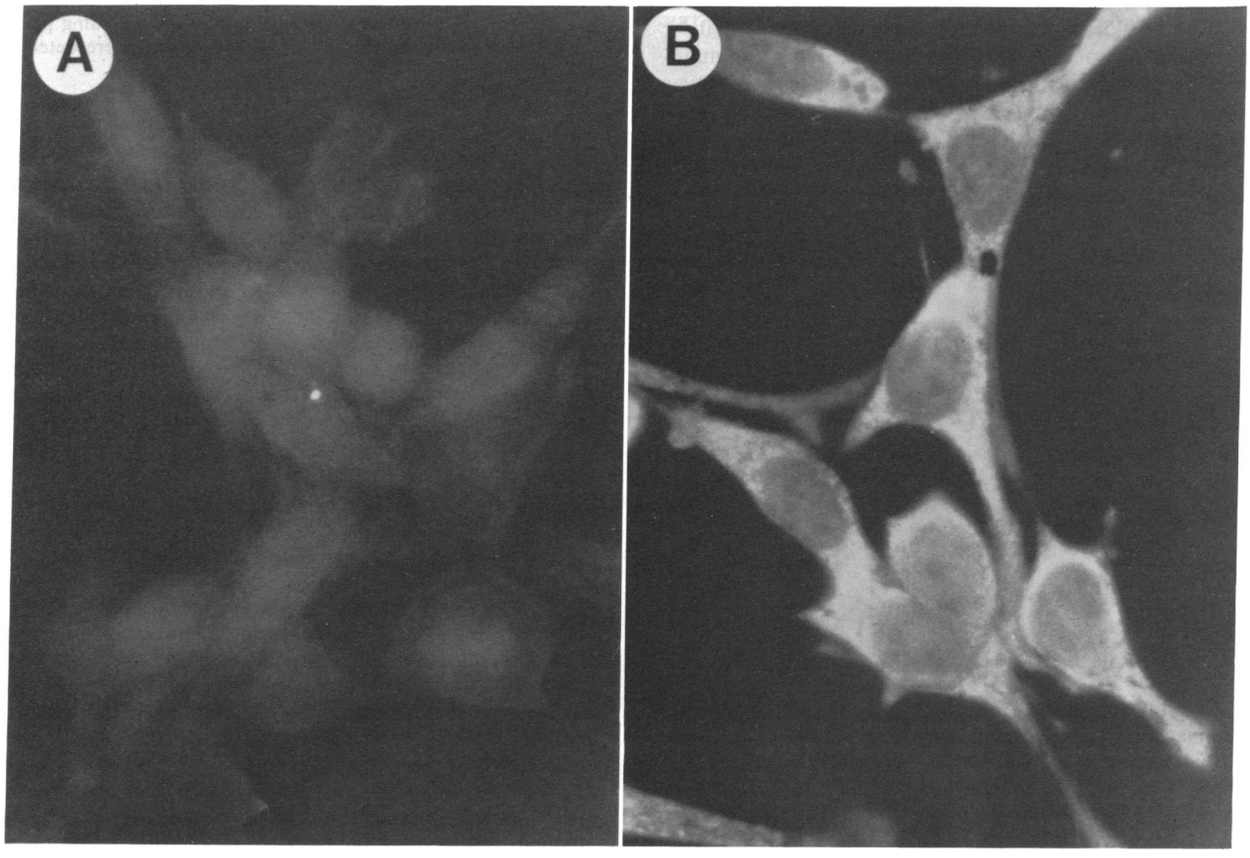


FIG. 6. Localization of $p70^{TRK}$ in *TRK*-transformed NIH 3T3 cells by indirect immunofluorescence microscopy. *TRK*-transformed NIH 3T3 cells (clone 106-63) were fixed with methanol and stained with either preimmune rabbit IgG (*A*) or IgG molecules purified from rabbit anti-$p36^{TRK}$ antisera (*B*) followed by fluorescein-conjugated anti-rabbit IgG as the second antibody (28).

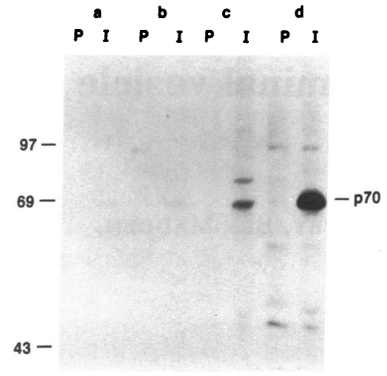


FIG. 7. Subcellular localization of p70$^{TRK}$ (p70). [$^{35}$S]Methionine-labeled *TRK*-transformed NIH 3T3 cells (clone 106-63) were disrupted in hypotonic medium, fractionated into nuclei (lanes a), nuclear-associated membrane (lanes b), cytoplasmic P-100 (lanes c), and particulate S-100 (lanes d) fractions as described (26). Each fraction was immunoprecipitated by using either preimmune (lanes P) or anti-p70$^{TRK}$ antisera (lanes I). Immunoprecipitates were analyzed by electrophoresis on $NaDodSO_4$/8% polyacrylamide gels. Gels were exposed to Kodak XAR films for 2 days. Molecular weight markers, shown $\times 10^{-3}$, were those described in the legend to Fig. 2.

tures characteristic of tyrosine-protein kinases (9). In this study, we have identified p70$^{TRK}$ as the gene product of the *TRK* oncogene and provided biochemical evidence indicating that this protein possesses protein kinase activity specific for tyrosine residues. We have also shown that under *in vivo* assay conditions, p70$^{TRK}$ is phosphorylated in tyrosine residues, a feature characteristic of most tyrosine-protein kinases that is thought to be involved in the modulation of their catalytic activity.

The tyrosine-protein kinase gene family encompasses two distinct classes of genes: those represented by the prototypical v-*src* oncogene and whose products are cytoplasmic proteins and those that code for transmembrane proteins, some of which have been shown to be transforming alleles of growth factor receptor genes (1). Whereas the v-*src*-like oncogenes possess a highly promiscuous kinase activity capable of phosphorylating tyrosine residues in multiple cellular proteins, those derived from growth factor receptors have a much more restricted target range (22, 24, 25–27, 29). In this study, we have shown that NIH 3T3 cells transformed by the *TRK* oncogene do not exhibit significantly elevated levels of phosphotyrosine residues. Whereas nucleotide sequence analysis has predicted that this oncogene is likely to be derived from a gene encoding a transmembrane receptor (9), its product, p70$^{TRK}$, is primarily a cytoplasmic protein. These observations raise the possibility that the limited substrate specificity of the *TRK* oncogene, and perhaps of other receptor-derived oncogenes, might be an intrinsic property of their tyrosine-protein kinases.

Malignant activation of tyrosine-protein kinase genes is thought to occur by mutations that deregulate their enzymatic activity. In the case of the *TRK* oncogene, it is possible that tropomyosin may confer a certain configuration to the tyrosine-protein kinase domain that allows it to function in a deregulated ligand-independent fashion. A similar model has been proposed for the malignant activation of the v-*erb*B oncogene (reviewed in ref. 30). However, preliminary results from our laboratory indicate that unlike p70$^{TRK}$, the gene product of the normal *TRK* protooncogene is a transmembrane protein (R. Oskam, D.M.-Z. and M.B., unpublished observations). Therefore, tropomyosin sequences might have played an additional role in the activation of the *TRK* oncogene by allowing the kinase domain of p70$^{TRK}$ to interact with certain cytoplasmic substrates whose unscheduled phosphorylation may trigger neoplastic transformation. The studies described in this report should facilitate future efforts aimed at identifying the physiological substrate(s) of p70$^{TRK}$, a necessary step to understand the molecular pathways by which the *TRK* oncogene induces malignant transformation.

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